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Nanomaterials in the Environment Acquire an "Eco-Corona" Impacting their Toxicity to *Daphnia Magna*—a Call for Updating Toxicity Testing Policies

Fatima Nasser, Julia Constantinou, and Iseult Lynch**

Nanomaterials (NMs) are particles with at least one dimension between 1 and 100 nm and a large surface area to volume ratio, providing them with exceptional qualities that are exploited in a variety of industrial fields. Deposition of NMs into environmental waters during or after use leads to the adsorption of an ecological (eco-) corona, whereby a layer of natural biomolecules coats the NM changing its stability, identity and ultimately toxicity. The eco-corona is not currently incorporated into ecotoxicity tests, although it has been shown to alter the interactions of NMs with organisms such as *Daphnia magna* (*D. magna*). Here, the literature on environmental biomolecule interactions with NMs is synthesized and a framework for understanding the eco-corona composition and its role in modulating NMs ecotoxicity is presented, utilizing *D. magna* as a model. The importance of including biomolecules as part of the current international efforts to update the standard testing protocols for NMs, is highlighted. Facilitating the formation of an eco-corona prior to NMs ecotoxicity testing will ensure that signaling pathways perturbed by the NMs are real rather than being associated with the damage arising from reactive NM surfaces "acquiring" a corona by pulling biomolecules from the organism's surface.

1. Introduction

Nanomaterials (NMs) are particles with at least one dimension between 1 and 100 nm. Due to their small size, NMs possess novel and unique qualities that are not traditionally exhibited by bulk material of the same composition. NMs have been at the core of novel research for two decades and are incorporated into products spanning a range of industrial fields. For example, NMs properties are exploited in cancer research, such as the utilization of surface plasmon resonance properties of gold (Au) NMs; here the NMs are conjugated to antibodies complementary to antigens on cancer cells and are thereby internalized, following which oscillation of the Au electron cloud at a specific wavelength of light converts the absorbed light into localized heat to specifically destroy cancer cells.[1] Another example is exploitation of the antimicrobial properties of silver (Ag) NMs which undergo high dissolution to release Ag^+ ions[2] and where the dissolution site, rate (and thus toxicity) can be adjusted depending on the surface coating.[3]

Considering that NMs are becoming so widely used, their release into the environment is inevitable. Despite this, considerably less research has focused on the implications of NMs on environmental organisms, especially under realistic conditions, than on development of their applications. NMs may enter freshwater systems from industrial effluent, where, for example, the concentrations of zinc oxide (ZnO) NMs, widely used in sunscreens and paints, in river waters has been found to be as high as 150 ng L^{-1},[4] while gold (Au) NMs excreted following use in medical applications into surface waters have been predicted to be 470 pg L^{-1}[5] With deposition of NMs into environmental waters increasing, concerns regarding the potential for toxicity posed by NMs have demanded action, although the standard testing approaches have not yet been fully updated for use with NMs to take account of their enormous reactive surface areas. Thus, providing ecotoxicity results based on realistic scenarios, taking into account the natural constituents of freshwaters including biomolecules released by the test organisms themselves, and their impact on NM surface (corona) and toxicity, is still an under-investigated area and caveats for NMs ecotoxicity assessment are detailed below.

Scientific understanding of the importance of biomolecule binding to NMs, and the formation of the protein (and later

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biomolecule) corona has been increasing over the last decade.[6,7] Indeed for cellular toxicity studies, addition of serum proteins to cell culture medium is standard in order to feed the cells, but also serves the role of coating the NMs and reducing their surface energy and damage to cells, allowing NM toxicity to be assessed under realistic exposure conditions without unrealistic damage to cells due to the NMs pulling out biomolecules to reduce their surface energy.[8] Similar effects are observed during in vivo toxicity studies, where the NMs are typically aerosolized, and it is well recognized that immediately upon contact with the lung fluids a biomolecule corona forms, that dictates how the NMs are subsequently processed in the animal.[9] Previous work relating to the biological corona has investigated how proteins in blood serum affect NM stability and toxicity, and similar principals apply for the eco-corona. However, in ecotoxicity testing, the importance and role of the corona has yet to codified into the standard testing approaches, which were designed for dissolved chemicals, rather than dispersions of particles with large reactive surfaces.

A key limitation of current standardized test guidelines is that the complexity of real natural waters is not reflected in the recommended test media, which are simple salt solutions. Natural waters contain a wide variety of constituents including natural organic matter (NOM) from the degradation of vegetation, algae and bacterial biomass[10] with a large fraction being dissolved organic carbon (DOC) consisting of both humic and fulvic acids, as well as smaller fractions of carboxylic acids, amino acids and sugars.[11] DOC is a major modulator of the structure and function of lake ecosystems and information has been compiled from over 7000 lakes from six continents indicating that DOC concentrations range from 0.1 to 322 mg L^{-1} and that organic matter content is ever present in real environmental waters.[12] Natural fresh waters also contain bacteria and bountiful amounts of algae, and other organisms that "condition" the water by secreting biomolecules, as well as agricultural waste products such as fertilizers from farming[13] and constituents of urban wastewater effluent,[14] resulting in a complex soup of constituents, all of whom could potentially bind to NM surfaces forming an eco-corona.

As noted above, the reactive surfaces of NMs drive them to interact with their surroundings and bind to available biomolecules to form an eco-corona. Indeed, in the absence of biomolecules, the NMs will agglomerate strongly, again as a means to reduce their surface energy. The binding of environmental biomolecules and natural water constituents changes the NM surface properties and their identity, stability, subsequent interactions with organisms, and potentially their toxicity. This review explores the range of biomolecules available to form an eco-corona, and the impacts that acquisition of an eco-corona can have on the toxicity of NMs to organisms, utilizing *Daphnia magna* (*D. magna*) as a representative freshwater species. *D. magna* is widely used in toxicity testing due to its sensitively to pollutants whereby it acts as an indicator species, and its position in the food chain which makes it an important keystone species. It is worth noting, however, that the type of organism, its feeding approach, and its microbiome will all play a role in the nature and composition of the eco-corona that forms, and on whether the eco-corona mitigates NM toxicity or enhances it. The framework presented here, considering the types and sources of environmental molecules, and the range of modulatory effects these interactions can have on NMs toxicity, provides a basis for integrating eco-corona as part of mainstream ecotoxicity testing, as has already been achieved for the protein corona in animal and human toxicity assessment.

1.1. Model Organism *D. Magna*

The model organism *D. magna* is a freshwater zooplankton that has been widely used for assessing the toxicity of bulk chemicals as well as NMs. *D. magna* is a filter-feeder, meaning that it is directly exposed to all pollutants present in the water, and is sensitive to low levels of pollutants, and thus has been at the heart of toxicity testing from the earliest days of environmental protection.[15,16] *D. magna* fulfils a central role within the ecosystem as they consume phytoplankton, which are responsible for forming organic compounds from dissolved organic carbon dioxide to maintain the aquatic ecosystem[17] as well as being prey for several types of invertebrates.[18] *D. magna* also undergoes asexual (parthanogenetic) reproduction when in ideal environmental conditions, which ensures the offspring are genetically identical, ultimately decreasing variation in experimental studies,[19] but also providing a strong indication of the presence of pollution when the exposed organisms produce male offspring and resting eggs.[20,21] Several studies pertaining to the toxicology of chemicals and NMs have used *D. magna* as the indicator species due to the variety of end-points that can be measured when exposed to a stimulus, including heart rate,[22] appendage movement,[23] brood number release,[24] male neonate production,[20] ROS formation,[25,26] morphological deformation,[27] spin rate,[28] and moulting pattern.[26,29,30] These studies are conducted in a range of approved biological media, although the medium is generally free of any biological material such as NOM or biomolecules such as proteins or polysaccharides that would naturally be present in real environmental scenarios, at least at the outset.

1.2. Biomolecules in Natural Waters and their Interactions with NMs

Despite it being widely recognized that NOM is a sink for a range of pollutants, including metals and organics, and thus influences their bioavailability,[31–33] interactions with NOM are not considered as part of standard toxicity testing, nor indeed in modeling of pollutant fate and behavior. This becomes especially problematic for NMs, where their enormous surface area, and sizes similar to those of NOM complexes in aquatic systems (size partitioning of dissolved NOM samples using asymmetrical flow field flow fractionation has shown a predominant size in the <5 nm size range[34]), means that it is no longer a question of NOM removing the pollutants from solution, but rather that the NOM binds to the surface of the NMs forming NM-NOM complexes, or a NOM-corona, thereby altering the NM surface properties and subsequent interactions. Other constituents of the aquatic environment, including exopolysaccharides secreted by bacteria and biofilms, and proteins and other biomolecules secreted and excreted by aquatic organisms, will also compete with NOM, depending on their affinities for the NM

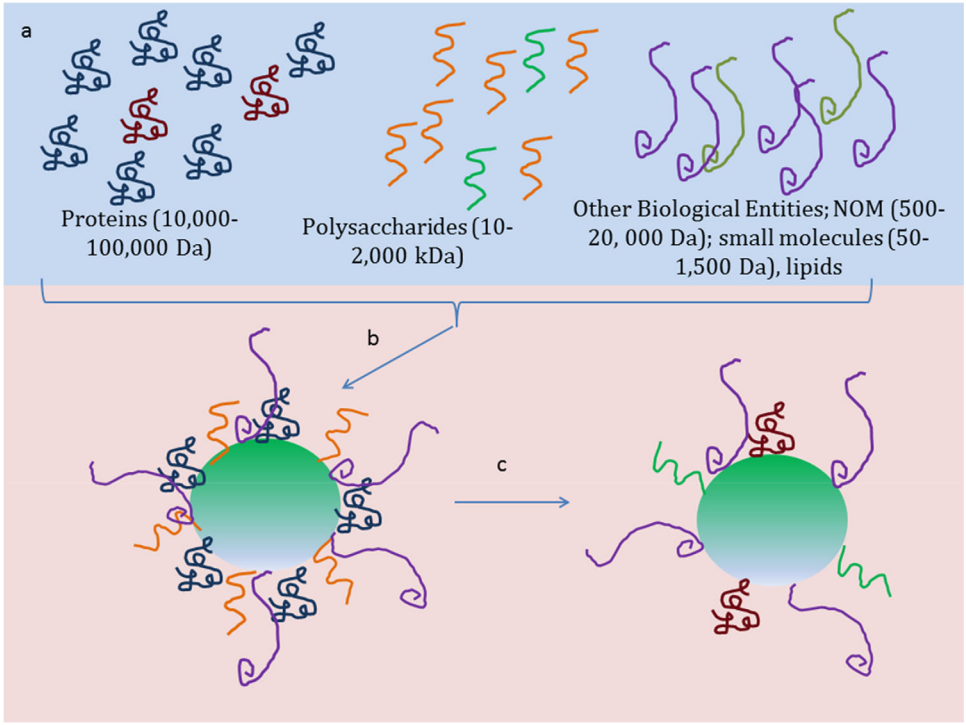


Figure 1. a) Biomolecules found in environmental waters such as proteins, polysaccharides and other biological constituents (including NOM, small organic molecules and lipids). b) Upon immediate exposure to NMs (green spheres), biomolecules of higher abundance bind to the NM surfaces first, which are then c) displaced by biomolecules with a higher affinity for the NM surfaces (also known as the Vroman effect[35,36]). This binding reduces the surface energy of the NMs, and provides a biological surface that can be "recognized" by cellular receptors.

surface. As per the well-known Vroman effect,[35,36] biomolecules in high abundance may bind initially to be subsequently replaced by molecules with a higher affinity for the NM surface, as shown schematically in **Figure 1**; evolution of the NMs corona in this manner can result from NMs uptake and transport to new locations with different biomolecule availability,[37] or from cellular secretions in response to the NMs presence which can alter the corona composition.[38] Indeed, evolution of the NM (eco)corona is an area of active research, both experimentally and through development of predictive models based on affinities.[39]

These biological and environmental constituents, whose molecular weights span from 10–2 000 000 Da, have the ability to adsorb onto NM surfaces and create a layer around the NM termed the "corona," or "eco-corona" to emphasize that it is composed of environmental biomolecules. This acquired layer of biomolecules changes the NM identity, stability, uptake into and toxicity to *D. magna*, which will be discussed in more depth in the sections below.

1.3. Toxicity of NMs to *D. Magna*

Several NMs have been studied in terms of their toxicity toward *D. magna,* mostly using the standard (OECD 202) short-term immobilization test[40] which investigates toxicity in terms of immobilization (mortality) without considering explicitly the mechanism of effect. Due to the filter feeding nature of *D. magna,* which takes up particulates from the water column based on size and consistency,[18] NMs are ingested alongside water, so that the gut of *D. magna* is exposed to the NMs, which may be endocytosed by gut cells inducing a range of responses including apoptosis, DNA strand breakage, mitochondrial swelling and dysregulation, all of which are accompanied by an increase in the amount of toxic reactive oxygen species (ROS).[41–44] The diversion of energy to the synthesis of additional antioxidant defense mechanisms to combat the excess ROS has been suggested to explain the observed inhibition of other secondary yet important functions such as moulting,[30] that can also lead to mortality as a side effect of ROS formation. Thus, most of the NM-related toxicity reported to date suggests that ROS overload is the primary mechanism leading to a cascade of events resulting in mortality. Interaction of NMs with *D. magna* can also cause toxicity even before ingestion, where NMs are able to bind to the surface of *D. magna* weighing the organism down so that it does not reside in the water column where the most amount of food exists. NMs can also bind to appendages resulting in deformation of swimming patterns and decrease in oxygen uptake.

D. magna utilizes a combination of peristaltic motion of its microvilli and the positive pressure from newly ingested food to push ingested matter through the gut, with the latter being the dominant force.[18] It has been shown that in the absence of newly ingested food, which is needed to push out previously ingested NMs, a residual amount of Polystyrene (PS) NMs remained within the gut of *D. magna* causing a decrease in the amount of food (*Cholera vulgaris)* that they subsequently consumed.[45]

Table 1. NMs used in various industrial processes and their corresponding acute, half-maximal effective concentrations (EC_{50}) toward *D. magna*, determined in biomolecule-free media unless otherwise indicated.

NM	Industrial process	Reported acute EC_{50} value for *D. magna*	Reference
Copper oxide (CuO)	Semiconductors, heat transfer fluids, intrauterine contraceptives	31 nm, acute 48-h OECD 202 test: 4 mg CuO /L	[46,47]
Silver (Ag)	Antibacterial, wound dressings and coating of surgical instruments	220 nm, acute 48-h OECD 202 test: 3.67 μg L^{-1}	[48,49]
Zinc oxide (Zn)	Paints, gas sensors, sunscreens and personal care products	20 nm, acute 48-h OECD 202 test: 0.622 mg L^{-1}	[50–52]
Titanium dioxide (TiO_2)	Catalytic Converters	21 nm, (composed of: 80% anatase/20% rutile), 48 h OECD 202 test: >100 mg L^{-1}	[53,54]
Polystyrene (PS)	From the degradation of bulk plastic	100 nm, Amino-functionalized PS, 24-h OECD 202 test: 0.0258 mg mL^{-1} 100 nm, 24-h OECD 202 test with 6 hr conditioning and 6 hr NM incubation prior to exposure: 0.0189 mg mL^{-1}	[45]
Au	Medical applications and cancer therapy	25 nm, Positively charged spheres, 24-h OECD 202: 6.11 μg L^{-1}	[26]
ZnO	Inclusion in rubber, ceramics and paints	5 nm, PVP capped 24-h OECD 202 test: 0.055 mg L^{-1}	[55]
CeO	Solid oxide fuel cells, catalysts, UV absorbants	5 nm, PVP capped 24-h OECD 202 test: 0.193 mg L^{-1}	[55]

This reduced feeding inclination may, over the longer term, decrease the amount of nutrients acquired by *D. magna* such that reduced nutrition may be a mechanism of NM-induced mortality. The presence of an eco-corona around these PS NMs resulted in enhanced retention of the NMs within the gut which could be due to enhanced binding to the gut wall.[45] The additional eco-corona coated NMs retained within the gut of *D. magna* increases the overall body burden, requiring more energy to maintain basic regulatory mechanisms such as swimming. NMs also have a high propensity to physically bind to *D. magna* as a means to acquire a biological surface coating; for example, TiO_2 NMs have been shown to bind to appendages resulting in changes in the swimming pattern and ultimately affecting moulting due to the diversion of energy required to maintain swimming.[30]

Table 1 provides a variety of examples of NMs, their industrial processes, and reported half-maximal effective concentration (EC_{50}) toxicity values toward *D. magna*. It is worth highlighting that the majority of these studies were conducted without the presence of biomolecules (unless otherwise stated), so that these values may be altered under realistic conditions.

1.4. Current Protocols for NM Ecotoxicity Testing

The organization for economic cooperation and development (OECD) has developed two specific test guidelines for measuring toxicity of chemicals (which includes NMs) toward *D. magna*. The OECD 202 is the acute immobilization test, which measures mortality of *D. magna* in the form of immobilization between 24 and 48 h. The longer-term OECD 211 chronic reproduction test looks at reproduction of *D. magna* spanning 21 days. Both protocols provide stringent instruction on exposure conditions such as test solutions, conditions of exposure and exposure duration, and are currently being updated with specific adaptions for NMs, taking account of the need to ensure that the NMs remain in the water column in order to ensure exposure, and other specific adaptions. There are several stark differences when investigating the toxicity of NMs compared to the toxicity of molecular chemicals for which these toxicity tests were originally designed. Chemicals dissolve into solution (medium) whereas NMs exist as a suspension causing them to have an ever-present, highly reactive surface,[56] making them prone to adsorbing organic constituents present in environmental waters such as biomolecules to their surface in order to lower their surface energy. By contrast, chemicals have no true surface for any biomolecules to bind to, although as noted above they can, and often do, sorb to NOM, thereby reducing their bioavailability. This layer of biomolecules or organic substances pulled from the environment onto the NM surface is known as the "eco-corona."

Currently, the OECD guidelines for the aforementioned tests do not require environmental or biological constituents such as NOM or biomolecules within their test media, which is not reflective of true environmental scenarios. It can be argued that the lack of ecological constituents would result in obtaining the worst-case scenario toxicity due to the high reactivity of the NM surfaces, but in biomolecule-free medium the NMs would very quickly acquire biomolecules directly from any organisms they come into contact with, and thus any toxicity data obtained would be reflective of this although without accounting for the spontaneous corona formation in the interpretation of the data. Indeed, it is difficult to remove all traces of NOM as even after using ion exchange to remove the majority of NOM, drinking water itself still contains between 2–10 ppm of NOM so that there are always traces of biological matter.[11] More importantly, organisms condition their surrounding medium very rapidly (minutes) and thus even if there are no biomolecules present in the medium at the start of the test, there will be within minutes, the nature of which will depend on the severity of the toxicant and its concentration, which will interact with the NMs, thereby dynamically altering the system during the test, confounding the results.[57] Here, we aim to bring a new perspective on the importance of the eco-corona for reliable ecotoxicity testing of NMs, and provide a framework within which to evaluate the modulating effect of the eco-corona on NMs toxicity, utilizing *D. magna* as the demonstration case. We propose that inclusion of biological matter

(whether secreted from the test organism via medium conditioning or representing those present in environmental waters) should become a prerequisite for ecotoxicity testing, as indeed serum proteins are for in vitro and most in vivo toxicity testing.

2. The Eco-Corona

NMs released into the fresh waters will inevitably interact with the vast array of biomolecules present in freshwater. Freshwaters contain runoff from vegetation resulting in the deposition of proteins, polysaccharides, and lipids within these waters, which can then be utilized for their amino-acids, monosaccharides, and fatty acids by organisms to support growth and development.[58] A range of organisms, from the simplest bacteria to complex biofilms and aquatic dwelling plants and animals secrete biomolecules into their surrounding medium. These secretions are collectively known as "conditioning" of the medium (further explained in Section 2.2) and are an important source of biomolecules to the surrounding environmental waters.

NMs have a high surface reactivity and therefore rapidly adsorb some of these biomolecules onto their surface in order to try to reduce their surface reactivity. Which biomolecules from the available soup that bind depends on both the biomolecule abundance and the affinity for the NM surface.[35] Biomolecule binding to the NM surface is driven by a range of forces including electrostatic, hydrophobic, entropic due to release of water or small ions, and indeed, some molecules may not bind directly to the NM surface, but instead bind to other biomolecules present in the eco-corona.[59] Initially proteins which are higher in abundance bind to NM surfaces and then are displaced by less abundant yet higher affinity proteins.[60,61] It has been evidenced that in blood plasma or serum, human serum albumin (HSA) dominates NM surfaces initially and subsequently becomes displaced by higher affinity proteins such as apolipoprotein (A-I) which are present in lower abundance and have slower binding kinetics. This phenomenon can be explained by the Vroman effect.[35] Similarly, it has been shown that different environmental species are able to adsorb and displace others on the surface of 20 nm TiO_2 NMs, where humic acid adsorbs tightly to NM surfaces to form part of the eco-corona while smaller acidic molecules such as ascorbic and citric acid bind weakly and can easily be displaced by humic acid.[62] Interestingly humic acid does not displace the protein bovine serum albumin (BSA) but both are able to co-adsorb onto TiO_2 NM surfaces so that binding affinity to NM surfaces is dependent on the different functional groups interacting.[62] The most abundant biomolecules in aquatic environments are NOM (with > 10 mg of C per L); polysaccharides which are usually deposited into fresh waters from fallen tree leaves and vegetation; and proteins which comprise 24–49% of TOC[63] (from 158 mg L^{-1}), each of which has a high propensity to bind to NMs and influence their toxicity.

NOM has been shown to create an eco-corona around citrate-coated Ag NMs, although the NOM-corona is largely dependent on the composition of the original NOM (this differed between two tested lakes).[64] The NOM corona was found to be rich in nitrogen and sulfur containing compounds and had preferential binding for high molecular weight (MW), highly unsaturated molecules with a high number of oxygenated groups, due to selective absorption driving binding,[64] though this is of course contingent on the intrinsic NM properties. Higher MW fractions of humic substances are also preferentially adsorbed by mineral particles over smaller fulvic acids and that the adsorbed amount of humic acid gradually increased with increasing humic acid concentration in the surroundings.[65]

NOM has also been shown to readily interact with diamond NMs forming an eco-corona in a manner that is dependent on the NOM to NM ratio (with NOM concentrations ranging between 0 and 30 mg L^{-1}) with the resulting eco-corona influencing the surface charge and NM stability. High NOM to NM concentrations resulted in agglomeration and increased NM hydrodynamic diameter.[66] At low NOM to NM ratios, the NMs only marginally agglomerated but retained their positive zeta-potential due to the non-uniform absorption of NOM molecules leading to attractive electrostatic interactions between oppositely charged regions on adjacent NM surfaces. This positive charge allowed for enhanced interaction between the NMs and the negative charge on the membrane of the bacterium *Shewanella oneidensis*, leading to enhanced toxicity compared to NMs without the NOM corona.[66] The corona that forms due to the absorption of NOM onto NM surfaces thus differs depending on the specific constituents of the NOM present in the medium as well as the dynamic and competitive adsorption of NOM molecules on the NM surface.

2.1. Release of Biomolecules (Medium Conditioning) by Organisms such as *D. Magna*

In addition to the NOM derived from decaying plant and animal matter, organisms such as *D. magna* release biological material into their surroundings as part of their normal filtering of water; this is known as "conditioning" of their surroundings. Cells on the outermost layer of organisms secrete proteins into their surroundings for a number of reasons such as the release of hormones (enzymes)[67] or antibodies for the purpose of internal regulation of the organism and also for cell-to-cell or organism-to-organism communication as a defense mechanism. Organisms such as *D. magna* also condition their surroundings by releasing substances called kairomones which trigger defense mechanisms in their prey[68] so that the organisms themselves are continually altering the concentrations of biological matter in their environment (**Figure 2**). *D. magna* also release proteins into their environment, for example, enzymes such as chitinase and chitinobase. These degrade the polymer chitin, which is the main component of the exoskeleton of *D. magna,* and are abundant prior to moulting, and released along with the moulting fluid into environmental waters.[69] *D. magna* also releases polysaccharides into their surrounding environment as a natural process of growth and development, whereby the chitin-based exoskeleton is released into the surrounding water.[70]

These secretions, released by *D. magna* as part of their normal functioning, shown schematically in Figure 2, thus include: 1) kairomones released by *D. magna* when over-crowded which signals stress within adjacent *D. magna* causing death and is therefore used as a population regulatory mechanism. Released kairomones are also used by algae as a signal to bunch together, making them more difficult to consume by *D. magna;* 2) *D. magna* also possesses an extensive gut microbiome where bacte-

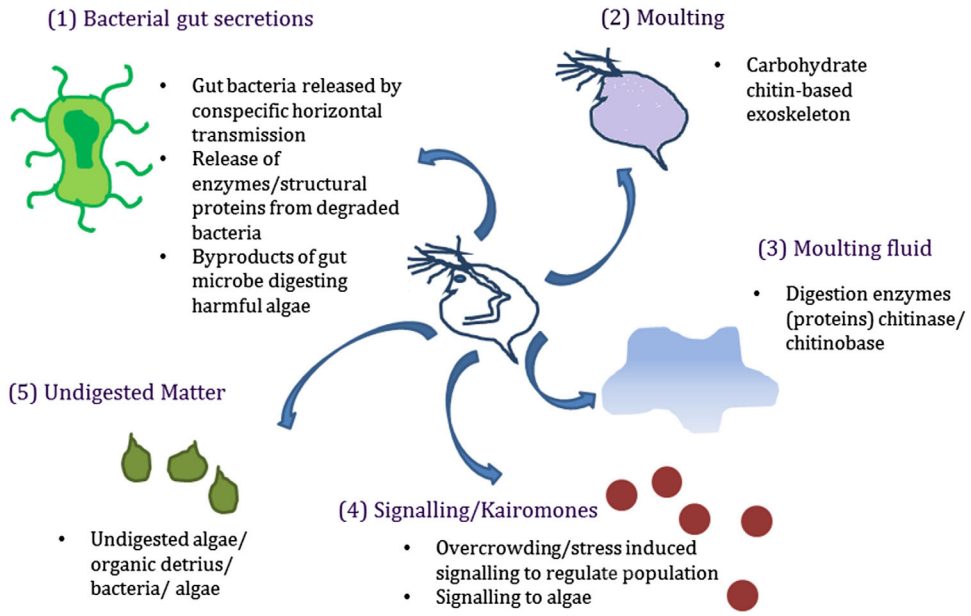


Figure 2. Various secretions from *D. magna* including: 1) bacterial gut secretions, 2) carapace from moulting, 3) moulting fluid, 4) surface-based secretions and 5) undigested or partially digested matter.

ria and bacterial secretions may be released through conspecific horizontal transmission resulting in the release of enzymes and proteins from expelled bacteria that are degraded. *D. magna* also releases by-products from the gut microbe digestion of harmful algae; 3) *D. magna* releases its chitin-based carbohydrate exoskeleton during their moulting process as well as; 4) the moulting fluid which contains digestive enzymes; and finally 5) *D. magna* will release any expelled matter after digestion including digested organic detritus and bacteria. These secretions are all potentially available for integration into the eco-corona.

These released biomolecules are continually being added to environmental waters and cause the organic matter concentrations of ecological systems to be dynamic. It is extremely likely that these secreted biomolecules can adsorb onto NM surfaces creating an evolving eco-corona thereby affecting the stability, identity and toxicity of the NMs toward *D. magna*. Indeed, it has been shown that both amino and carboxylic acid functionalized PS NMs and polyvinylpyrrolidone-capped ZnO and CeO_2 NMs agglomerate when incubated in conditioned medium, with longer incubation times leading to larger NM sizes which increased their uptake by *D. magna* and resultant toxicity.[45,55] Given that zooplankton such as *D. magna* feed on algae, it is important to understand the potential role of algal secretions also in NM eco-coronas. While not comprehensively studied to date, early work on the eco-corona indicated that the contribution of algal medium conditioning to NM stability and corona formation was minimal, at least in terms of secreted proteins.[45] Further research, focusing on secreted polysaccharides, for example, may shed new light on this aspect, and will also enable consideration of food-chain effects, for example.

As a result of these organism secretions, any biomolecule free-medium present at the beginning of the OECD tests will slowly have biomolecules added to it throughout the exposure duration, ultimately changing the experimental conditions throughout the process. It has been shown that *D. magna* neonates steadily secrete approximately 4.35 ug mL^{-1} per daphnid of protein in 6 h, including the release of proteins such as Type VI secretion system (74.8 kDa) and Sensor protein QseC (50.4 kDa).[45] It is important to note that the proteins released by *D. magna* may be different at different stages of life (such as a one day old neonate versus a seven day old juvenile) so that corona formation and ultimately the stability of the NMs and toxicity is dependent on the age of the *D. magna* secreting the proteins. **Table 2** indicates the different proteins secreted by one day and seven day old *D. magna* (this work has been previously published in Briffa et al., 2018, Supporting Information.[51] Clearly, the proteins and biomolecules comprising the eco-corona are dependent on what proteins are present in the medium, which will be contingent on the age of the *D. magna*.

Table 2. Mass spectrometry identification of the proteins released by 1 or 7 day old *D. magna* (ten daphnids conditioning 2 mL of high hardness (HH) Combo medium for 24 h in each case).

One-day-old *D. magna* neonate	Seven-day-old *D. magna* juvenile
Serum albumin	Putative histone H3.2 (Fragment)
Putative Actin	Carbohydrate sulfotransferase (Fragment)
DnaJ subfamily C	Elongation factor 1-alpha
Hemoglobin subunit alpha	Carboxylic ester hydrolase
Lactotransferrin	Histone H3.3
Dynein heavy chain 6, axonemal	
Lysozyme malic enzyme (Fragment)	
Hemoglobin subnit beta	
Viral T-cell receptor beta chain T17T22 (Fragment)	

It was also observed that NMs acquire a corona during the incubation with *D. magna,* and that this contains different proteins compared to those determined on the NMs following exposure to conditioned medium.[71] Here, coronas formed on Au NMs incubated in tap water, *D. magna* conditioned (CTW) tap water, and tap water in which *D. magna* were present during the 24 incubation (DTW), were compared, and only a minimal amount (8 out of the 175 proteins present in the CTW and the 90 present in the DTW) of similar proteins existed on the coronas.[71] The higher number of proteins in the CTW conditioned medium compared to the DTW is reflective of the fact that the DTW corona is composed only of digestive fluid proteins, while the CTW one contains the full spectrum of secreted biomolecules, as indicated in Figure 2 above. Note that adult *D. magna* from a wild strain cultured in the lab for >100 generations were used in these experiments, which likely explains the larger number of proteins identified in the conditioned water compared to those in Table 1.

A comprehensive functional and biochemical inventory of the proteome of *Daphina pulex* (*D. pulex*) found that extracellular proteins account for as high as 8–10% of total proteins such that a strikingly large proportion of the entire proteome is secreted extracellularly[72] and available to interact with NMs in the environment. It is therefore important to acknowledge that these proteins have the ability to form an eco-corona which influences toxicity, and that this secretion and corona formation will happen during exposure under standard test conditions also. Indeed, the study by Mattsson et al. (2018) using adult *D. magna* demonstrated differences in coronas formed in real time in the presence of *D. magna* compared to those formed using preconditioned medium with no organisms present, showing much richer corona compositions in the conditioned medium.[71] 28% of the proteins identified in the *D. pulex* proteome were involved in "binding activities"[72] indicating that their very nature predisposes these proteins for binding, including to NMs forming eco-coronas. This highlights the importance of altering the current standard toxicity protocols to account for secreted proteins, and our recommendation is to include a 24-h age-matched *D. magna* conditioning step, followed by incubation of the NMs in the conditioned media (e.g., for 6 h) and then exposure of the corona-coated NMs to fresh organisms. Age-matching is important given the large differences in the numbers and types of proteins that will be present at different life stages.

2.1.1. Kairomone Secretion by D. Magna and their Influence on NM Toxicity

The characterization of kairomones released by *D. magna* is extremely limited to date; while release of kairomones is traditionally thought to provide a signal to influence defense responses,[73] these biomolecules also play an important role in regulation of survival of *D. magna* colonies. To highlight the importance of the release of kairomones by *D. magna* on the organisms themselves, a study of kairomone governed population regulation was conducted. *D. magna* prefers living in groups, the communication within which is believed to be governed by the release of kairomones. The survivorship of 5 *D. magna* neonates (1 day old at start) in 250 mL of high hardness (HH) Combo medium was compared to that of isolated *D. magna* neonates each individually cultured in 50 mL of HH Combo medium and to isolated *D. magna* individually cultured in 50 mL of conditioned HH Combo medium that came from a 1L running culture that had previously contained 20 *D. magna* (15–20 days old), by monitoring survivorship for 120 days ($n = 25$ daphnids for each condition (five jars with 5 *D. magna* each in the case of grouped neonates). **Figure 3** shows that *D. magna* grouped together survived for the longest duration, reducing to 50% survivorship at approximately 85 days and with the last organism mortality occurring at 120 days, compared to single neonates in plain medium reaching just 50% survivorship at approximately 65 days and the last neonate mortality occurring before 90 days. Interestingly, the single neonates exposed to conditioned medium (containing kairomones previously released by *D. magna)* had a much higher survival rate than the isolated organisms in plain medium, maintaining 50% survivorship past 70 days with the last organism mortality occurring past 110 days, results which are similar to those of the neonates that had been grouped together (Figure 3), indicating the importance of secreted biomolecules to organism health.

This increased survivorship of single *D. magna* in conditioned medium (previously inhabited by other *D. magna*) compared to single neonates in plain medium is likely due to the presence of kairomone secretions by previous *D. magna*. *D. magna* communicates through kairomone secretion as the level of kairomones dictates population survival, for example, when numbers of *D. magna* are too high which may lead to lack of resources such as food, it promotes death in the culture. Alternatively, when kairomone levels are too low and the population is at risk of survival, it promotes a stress response to switch to sexual reproduction leading to production of diapause eggs to ensure the future survival of the population as the current population dies out. Single females exposed to medium having previously contained a healthy population size and thus a suspected healthy level of kairomones survive significantly better than single females exposed to control medium. This data is important as it shows that *D. magna* secretes biomolecules other than the already well-established proteins and carbohydrates, which will inevitably compete for space at the NMs surface to form part of the NMs corona, which may also influence toxicity tests by reducing the concentration of these key biomolecules that promote survivorship. These data also support the need for medium conditioning to be added to standardized OECD ecotoxicity tests relating to *D. magna*, as the absence of kairomones may make the test organisms generally less robust, and thus more susceptible to toxicants.

There is no concrete research to date investigating how secreted kairomones may impact NM toxicity toward *D. magna* themselves, or their prey, although this is a novel and emerging area of animal toxicity. Although the release of kairomones by *D. magna* are accepted and their results are profound, they are not mentioned by the current OECD protocols as entities that are able to adsorb to NM surfaces. Secreted kairomones promoting group survivorship may be "removed" from the medium by binding to NM surfaces and in the case of high density NMs (gold, silver, lead as examples) may then settle at the bottom of the exposure vessel and are therefore removed from the water column where *D. magna* primarily exist, resulting in increased mortality as a false-positive and could be mis-allocated to NM toxicity akin to

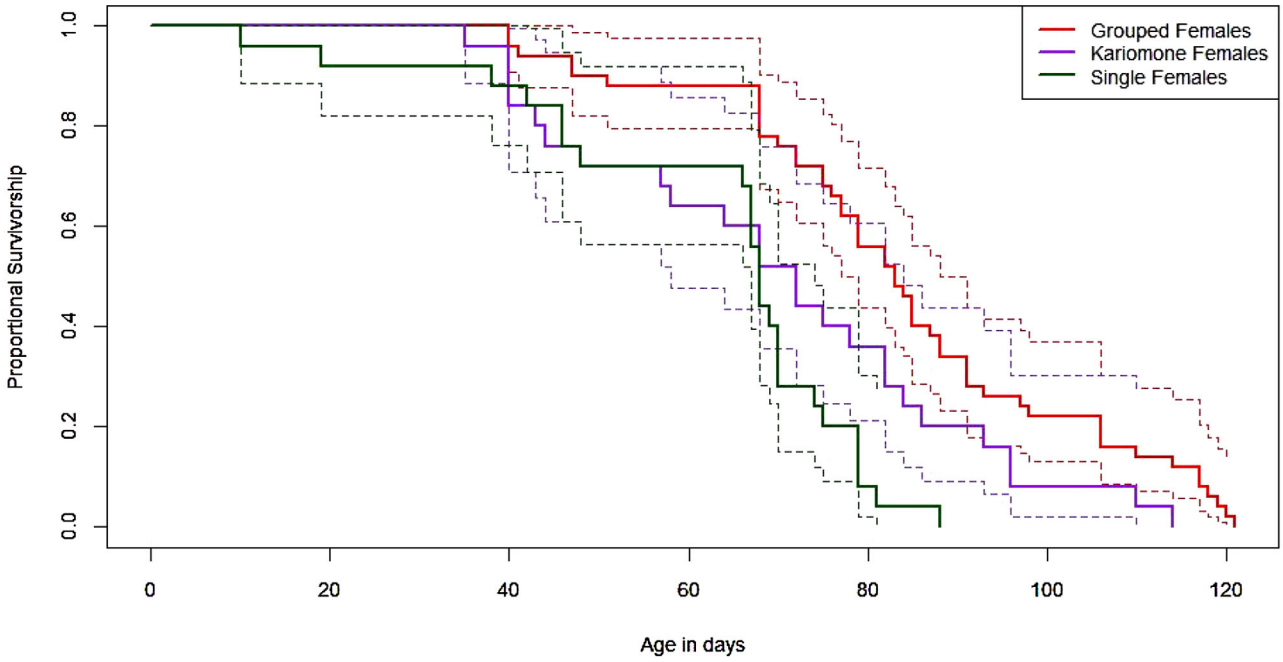


Figure 3. Survivorship of five grouped females (red), single females in plain medium (green) and single neonates in medium conditioned by previously containing 20 females (15–20 days old) (purple). Dotted lines represent Kaplan–Meier 95% confidence markers ($n = 25$ in each group).

the removal of proteins from media which has previously been shown with carbon nanotubes.[74]

2.2. Changes to the Eco-Corona Moving through the *D. Magna* Gut Passage

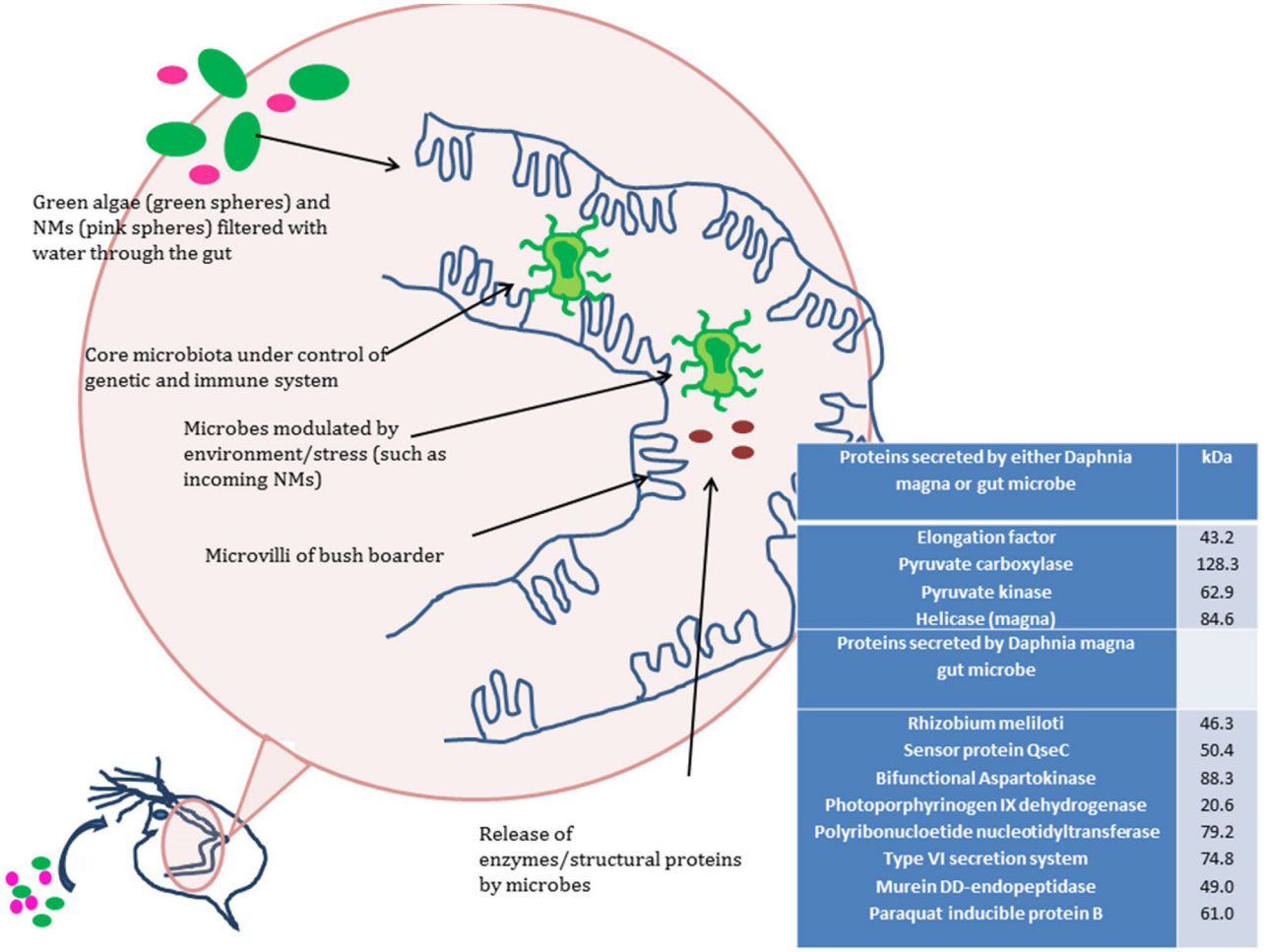
The NM eco-corona may further evolve once the NMs are consumed by *D. magna*, as the range of biomolecules and their affinities for the NM surface changes as the NM travels through the gut passage. Thus, exchange of biomolecules from the initial eco-corona with biomolecules released by the gut microbe bacterial community within the gut passage of *D. magna* may occur. The gut microbiota is a multi-layered structure consisting of both a core microbiota under the control of the genetic and immune systems as well as a flexible pool of microbes modulated by the environment[75] so that the microbiota present within the gut and their released biomolecules may differ in times of stress, for example, if NMs prompt ROS formation by the organisms. Microbiota act via symbiosis with *D. magna* and provide services such as enhanced food digestion,[76] uptake of nutrients,[77] and detoxification of harmful substances.[78] Microbiota residing within the gut microbiome also release biomolecules so that the total secreted biomolecules by *D. magna* belong to both *D. magna* itself as well as its hosted gut microbe. **Figure 4** shows a zoomed in version of the gut of *D. magna* and indicates the types of proteins that are released by *D. magna* neonates after 6 h of conditioning their surrounding medium and whether these are proteins released by the gut microbiome or secreted by *D. magna* itself.

The biomolecules released by microbiota may exchange with the adsorbed constituents on the eco-corona if those in the microbiome are in greater abundance or have a higher affinity for the NM surface than those biomolecules originally comprising the corona. This also means that NMs that are expelled from *D. magna* after egestion may have a completely different eco-corona to the NM that was originally ingested. Also, bacteria within the gut microbiome may also release biomolecules, which are expelled into environmental waters so that these biomolecules may bind to form the original eco-corona around NMs even before any NMs are ingested. It was found that 50 nm PS NMs incubated in 3-h *D. magna* conditioned medium for 1 h had bacterial proteins on their hard-corona released by the gut microbiome of *D. magna* (unpublished data). These proteins included signal transduction histidine used by bacteria to sense and respond to a wide range of environmental stressors,[79] as well as metallo-B-lactamase which are enzymes released by gram negative bacteria in digestion of toxicant drugs.[80]

2.3. NM Characteristics Influencing Eco-Corona Formation

2.3.1. Shape and Curvature

For NMs, diverse shapes can impact how biomolecules adhere to the surface, as curvature effects differ from one NM shape to another, as does the coordination of the atoms on the available surfaces, leading to different surface energies for different NM morphologies, which influences biomolecule binding.[81] The corona pattern formed around differently shaped Au NMs (25 nm spheres, and short (60 nm) and long rods (146 nm) both with 25 nm diameter) from *D. magna* conditioned medium (24-h conditioning by 10 *D. magna* neonates aged 1–3 days) of 5 mL of



Proteins secreted by either Daphnia magna or gut microbe	kDa
Elongation factor	43.2
Pyruvate carboxylase	128.3
Pyruvate kinase	62.9
Helicase (magna)	84.6
Proteins secreted by Daphnia magna gut microbe	
Rhizobium meliloti	46.3
Sensor protein QseC	50.4
Bifunctional Aspartokinase	88.3
Photoporphyrinogen IX dehydrogenase	20.6
Polyribonucloetide nucleotidyltransferase	79.2
Type VI secretion system	74.8
Murein DD-endopeptidase	49.0
Paraquat inducible protein B	61.0

Figure 4. A zoomed in schematic illustration of the gut of *D. magna* filtering water including green algae and NMs which pass through the gut (with microvilli in the bush border for absorbing nutrients), and core (immune system) and environmentally regulated microbiota. The figure also shows the specific proteins that are released by *D. magna* neonates after 6 h of conditioning the medium and indicates whether these proteins are released by the gut microbiota or *D. magna* itself.

HH Combo medium) results in significant differences in terms of the numbers and MWs of the bound proteins, as shown in **Figure 5**.

Figure 5 shows that differently shaped (sphere, short rod, and long rod) Au NMs incubated in *D. magna* conditioned medium all acquired different proteins in their coronas, as evidenced by polyacrylamide gel electrophoresis (SDS-PAGE) indicating that corona formation is morphology-dependent as they all had the same core composition and the same surface charge. It has previously been shown that these differently shaped NMs prompted different amounts of ROS formation in *D. magna* and that the *D. magna* were able to recover to different degrees from this; while the negatively charged Au NMs did not cause significant ROS formation, recovery, defined as the ability of the organism to return to steady-state ROS levels, was better when exposed to spheres compared to short rods.[26] Identical Au NMs with positive charges prompted a much higher degree of ROS formation with much poorer recoveries.[26] NMs of different shapes that were originally stable in fresh medium were found to agglomerate to different degrees in medium containing biomolecules specifically from *D. magna*; for example, long rods remained stable for up to 8 h whereas both spheres and short rods agglomerated, with size increasing up to fivefold, indicating that the presence of secreted biomolecules has a destabilizing effect on smaller NMs.[26] The effect of this agglomeration of the smaller spherical NMs, due to the destabilization resulting from the acquisition of the corona, caused an increase in *D. magna* mortality upon exposure. By contrast, Au NMs were found to be stabilized by *D. magna* both during direct exposure and following incubation in *D. magna* conditioned medium,[71] indicating that the consequences of the NM corona need to be determined for each type of NM for the moment, at least until sufficient data has been collected to allow development of predictive models for eco-corona formation, as is now being achieved for human serum/plasma coronas.[82,83]

With regard to the binding of NOM onto differently shaped NMs, there is somewhat more information on how NOM impacts the stability and shape of NMs,[84,85] rather than what fractions of NOM bind to NMs initially and following exchange. This is currently an under-investigated area in terms of understanding

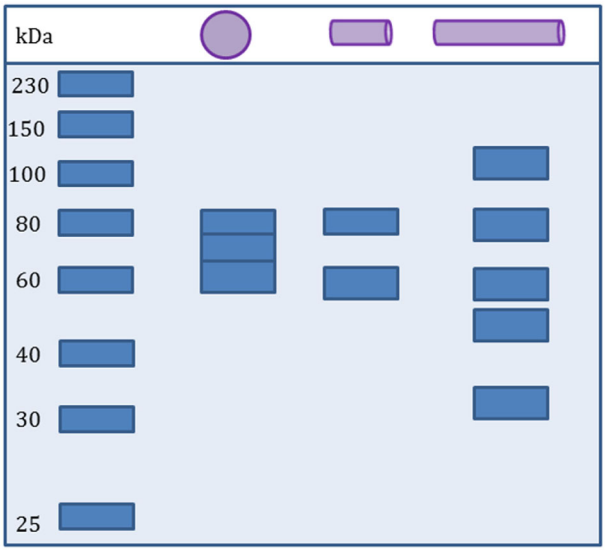


Figure 5. Schematic SDS-PAGE (with Coomassie blue and silver staining) of the proteins forming the corona around three differently shaped (sphere, short rod, long rod) negatively charged Au NMs that were previously incubated in 24-h *D. magna* conditioned medium.

and predicting NMs ecotoxicity, although studies in this direction are now appearing in the literature.[64,65]

2.3.2. Surface Charge

Surface charge also plays a role in the creation of the NOM eco-corona, although most studies to date have focused more on how NOM influences NM charge rather than what constituents of NOM bind and how NM charge governs this. One example shows that positively charged diamond NMs agglomerated when incubated in Suwannee river NOM and retained a positive zeta potential as NOM molecules adsorbed non uniformly causing oppositely charged regions on adjacent NMs to attract.[66] Selective binding of NOM from river water onto neutral or negatively charged Au NMs prevented agglomeration while NOM binding to positively charged Au NMs promoted agglomeration of the NMs. Thus, colloidal stability was linked to eco-corona formation which was regulated by the charge of the surface coatings.[86] More work is needed to investigate the nature of the sub-fractions of NOM bound in each case, in order to explain these findings, and to facilitate development of predictive models.

2.3.3. NM Size

The curvature of the NM is contingent on the NM size and affects the adsorption of biomolecules compared to their adsorption onto a flat object of the same composition.[59,87] It was shown that Au NMs of various sizes between 5 and 100 nm that were incubated individually with the common blood proteins albumin, fibrinogen, and insulin interacted well with each of these proteins and that the binding affinity of the proteins was dependent on the NM size.[88] The thickness of the protein corona was found to increase as the NM size increases as measured by fluorescence quenching. This may be because the curvature of smaller NMs may cause a decrease in binding of larger or more inflexible proteins.[88] This is likely to also be of importance for NOM coronas, where high molecular weight fractions such as Humic acids may have lower ability to bind to smaller NMs than smaller Fulvic acids, despite the Humic Acids being the larger fraction of NOM, that is, despite being more abundant.

2.3.4. Impact of Organism on Corona Formation

Coronas have been shown to be influenced by specific organism traits; for example, it was shown that 50 nm PVP-coated Ag NMs incubated in the plasma of the smallmouth bass fish creates a protein corona which increases in size over time (1–24 h). It was also shown that the constituents of the corona are influenced by the gender of the fish, whereby NMs incubated in male plasma have thinner coronas, while those that are incubated in female plasma have egg-specific proteins and have an especially large fraction of <20 kDa proteins which increased to >50% by mass after incubation for 24 h.[89] The presence of an eco-corona has been shown to impact on the uptake of 75 nm Ag NMs toward the earthworm *E. fetida*. Ag NMs have been shown to adsorb secreted native coelemocyte proteins to their surface and also to separately adsorb non-native FBS proteins to their surface. Coelmocytes preferentially take up Ag NMs coated in their own native proteins compared to those with a foreign corona.[90] The presence of a humic acid corona (at a concentration of 10 mg L^{-1} in the medium) around citrate coated Ag NMs was shown to ameliorate NM-induced toxicity in zebrafish. The mechanism of toxicity of the pristine NMs was binding of NMs to the gill membranes and thus interaction of the NMs with humic acid and formation of the eco-corona likely prevents membrane damage and limits the resulting oxidative damage.[91] The impact of NOM interaction with Ag NMs was found to be dependent on the NOM chemical composition; for example, sulfur and nitrogen rich NOM increased NM stability and decreased their toxicity toward the bacteria *Shewanella oneidensis MR-1*.[92] The affinity of the capping agent (PVP or citrate) for the NM, and how easily the capping agent could be displaced by NOM, were important factors influencing the effectiveness of NOM stabilization. Mechanisms hypothesized for the reduced toxicity of the NOM-corona coated Ag NMs included the NOM acting as a physical barrier preventing NM-bacteria contact or the corona acting as a ROS scavenger.[92] The presence of a humic acid eco-corona mitigated toxicity of hematite NMs toward the bacterium *Pseudomonas putida* and increased its EC_{50} from 24 to 4774 mg L^{-1}. The mechanism of toxicity determined by TEM showed that the surface bound humic acid prevented adhesion of hematite NMs to the bacteria and limited internalization.[93] It has also been shown that the presence of humic acid decreases the toxicity of graphene oxide (GO) toward zebrafish, where pristine GO induced damage to the chorion membrane and created excessive ROS. NOM was able to mitigate toxicity by reducing interaction between GO and the chorion and regulated the morphology, structure and surface charge of GO as well as altering the uptake of GO by the embryonic yolk cells.[94]

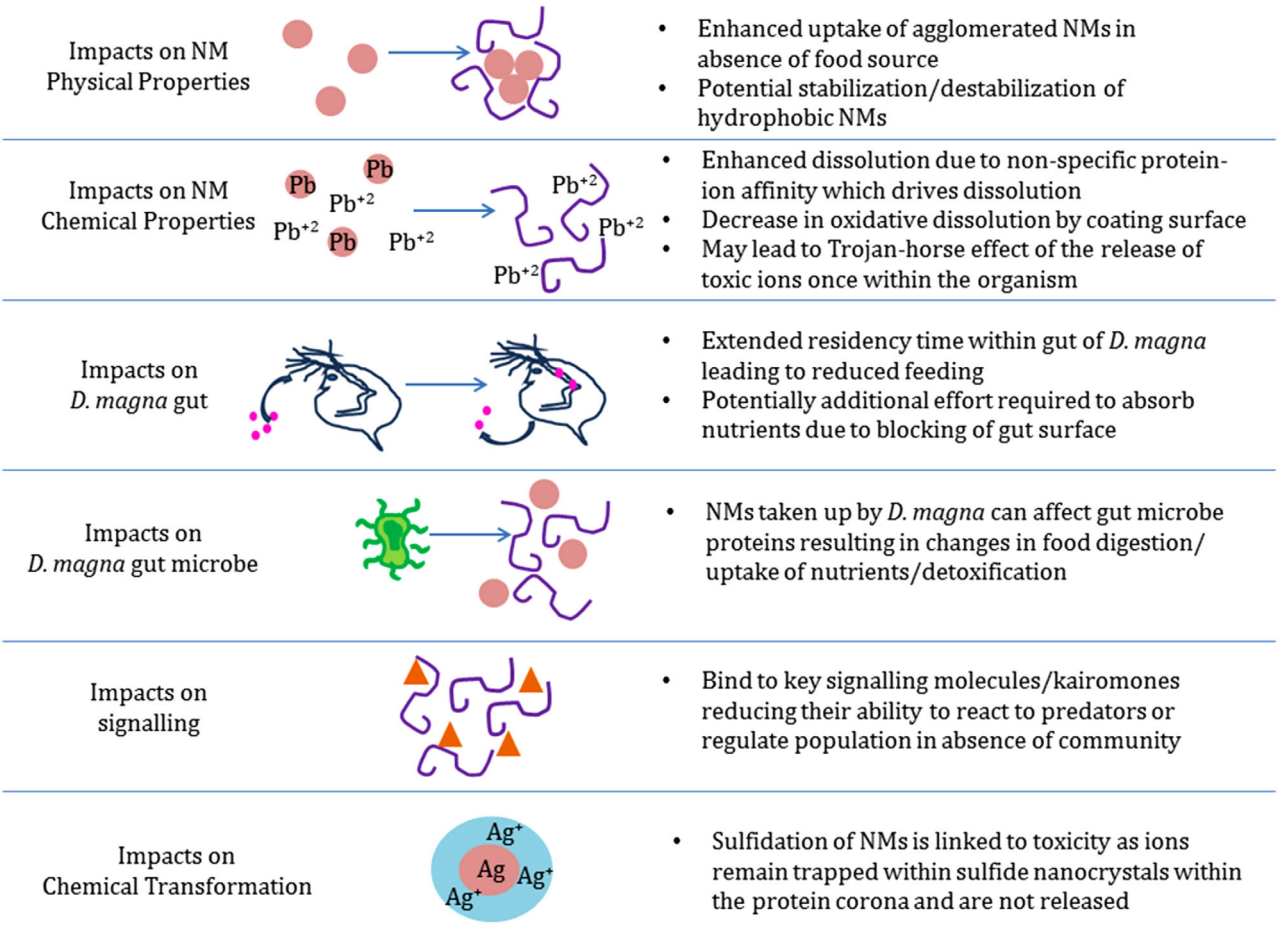


Figure 6. Possible mechanisms by which an acquired eco-corona can module the toxicity of NMs in environmental waters. The eco-corona has potential impacts: on NM physical properties influencing NM stability and uptake; NM chemical properties such as driving dissolution; on *D. magna* gut influencing residency time and absorption of nutrients; on the gut microbiome of *D. magna* influencing biomolecules released by gut bacteria; on signaling by binding to key signaling molecules such as kairomones and on; chemical transformations such as sulfidation of NMs trapping ions within the corona thereby influencing toxicity.

3. Effect of "Eco-Corona" on NM Toxicity to *D. Magna*

The presence of biomolecules or biological constituents such as NOM has the ability to create an eco-corona which can affect NM stability and therefore their uptake by, and toxicity, toward *D. magna*. By increasing the agglomeration of NMs and coating them with biomolecules, the eco-corona can enhance the attractiveness of NMs as a food source for *D. magna*, leading to enhanced toxicity.[45] On the other hand, NOM has been widely used as a dispersant of NMs, and so can reduce the agglomeration.[95] Biomolecules or NOM eco-coronas can also prompt other mechanisms of toxicity such as enhanced dissolution, "pulling out" toxic ions from NMs and increasing their bioavailability. Alternatively, formation of the eco-corona may slow oxidative dissolution processes, by blocking or delaying access of oxygen to the NM surface,[96] and can influence surface reactions such as sulfidation which is known to reduce Ag NM toxicity by preventing dissolution.[97] Eco-corona coated NMs may also lead to an extended residency time within the gut of *D. magna* and reduce their subsequent feeding or lead to additional energy required for *D. magna* to extract and absorb nutrients due to the blockage of the gut surface. NMs may also bind kairomones, reducing the ability for *D. magna* to sense predators or to regulate mechanisms such as population modulation. These mechanisms of toxicity are illustrated schematically in **Figure 6**, and are discussed in further detail below.

3.1. Eco-Corona Induced Agglomeration Impacting NM Uptake and Retention

Biological or environmental fluids that contain macromolecules such as proteins will cause a thermodynamically favorable exchange of biomolecules or synthetic ligands at the surface of the NM with biomolecules existing in the medium. This can cause a destabilization of originally monodisperse NMs causing agglomeration in the medium or conversely have a stabilization effect. Recently, it has been shown that NMs with a stabilizing shell

of polyethylene glycol (PEG), which usually remains monodisperse in the short term, agglomerate when incubated in vivo as measured by the aggregation index (the ratio of the absorbance at 620 nm to the absorbance at 520 nm)[38] and by DLS[98] in their corresponding media, indicating the exchange of biological mater with polymer molecules originally present at the surface of the NMs. It has also been shown that humic acid strongly adsorbs to 20 nm TiO_2 surfaces and easily displaces smaller acidic molecules such as citrate, which were originally used as a capping for the NMs but are much more weakly bound.[99]

An increase in NM size due to destabilization resulting from a corona formation is equally important when investigating toxicity toward filter feeders such as *D. magna* that take up particulates from the water column based on size and consistency.[18] Eco-corona agglomerated NMs may be closer in size to their natural algal food source of 1 µm[18] and therefore the presence of an eco-corona may have a profound effect on the uptake and thus the toxicity of the NMs. For example, PS NMs incubated with medium containing 435 µg mL^{-1} of proteins released by *D. magna* increased in size from 100 to 300 nm within 6 h of incubation,[45] and the degree of agglomeration was dependent on the incubation time of the NMs within the biomolecule containing medium. Incubation of NMs in protein containing medium effect NM size and longer incubation times led to a higher degree of agglomeration due to a longer amount of time for NMs to interact with proteins creating the final eco-corona.[45] NMs that had been incubated for a longer amount of time (24 h versus 6 h) in 6 h conditioned medium led to a decrease in the half maximal concentration value (EC_{50}) from 0.0189 to 0.0081 mg mL^{-1}, both of which were lower than the EC_{50} concentration of 0.0258 mg mL^{-1} with no proteins present. This is due to the presence of proteins creating an eco-corona around the NMs thereby destabilizing them and causing them to increase in size causing enhanced uptake and thus enhanced toxicity.[45]

Given the limited research has been done on the effect of the eco-corona on the toxicity of NMs toward organisms to date, the effect must be considered on a case-by-case basis depending on the organisms and its mechanisms of potential interaction with NMs. Generally, smaller NMs are said to be more toxic than larger ones when comparing on a mass basis, due to the larger available surface area to interact with the organism, and NMs less than 100 nm can enter cells of organisms such as gut epithelial cells, while NMs below 40 nm can potentially enter the nucleus.[100]

3.2. Influence of Eco-Corona on Toxicity toward *D. Magna* after Consumption

D. magna is a filter feeder, taking up particulates from the water column. Once food or particulates are taken up, it moves through the digestive system which is tubular and divided into three parts; the esophagus, midgut, and hindgut (Ebert).[18] The midgut is lined with epithelial cells and has a bush boarder protruding into the gut, lined with microvilli to increase the surface area for the absorption of nutrients. Peristaltic contractions push material forward through the gut, but more importantly the uptake of newly acquired food creates pressure to dispel already ingested material, which is also a route of secretion of biomolecules into the surrounding medium. In the absence of food, about 15% of the carboxylic acid (COOH) functionalized PS NMs that had been taken up remained within the gut though those that had been pre-incubated in conditioned medium containing proteins had an enhanced retention with 20% remaining within the gut.[45] This indicates that the eco-corona induced agglomeration results not only in enhanced uptake, but once consumed, drives a higher retention of the NMs within *D. magna* leading to enhanced toxicity and stress. As NMs pass through the gut, any original eco-corona biomolecules may be replaced by biomolecules released by gut microbe bacteria or enzymes secreted into the gut by *D. magna* and their absorption to NM surfaces may prevent them from carrying out essential processes such as food digestion or detoxification of harmful species.

ROS formation is an excellent indicator of organismal and cellular stress, and ROS production has been shown to be influenced by charge and surface ligand groups on NMs and effects may be ligand specific. The presence of ecological biomolecules have the ability to replace ligands on NM surfaces, creating a new surface coating as an eco-corona and therefore have the ability to influence ROS generation. The additional retention of NMs induced by the acquired eco-corona can cause toxicity at a chemical level in terms of ROS formation where *D. magna* have been shown to prompt a high degree of ROS production when exposed to a high concentration (EC_{40}) of positively charged 25 nm gold (Au) spheres, although the organisms are able to return to steady-state levels within 8 h post-exposure.[26] Interestingly, when 25 nm Au spheres were incubated for 6 h in 6-h conditioned medium (where *D. magna* had been allowed to release biomolecules into their surrounding media), the spheres agglomerated to >150 nm leading to increased mortality where the organisms do not recover from the amount of ROS produced in response to the NM uptake.[26] Extended residency times of eco-corona-bound NMs within the gut of *D. magna*, compared to those of bare NMs, had the ability to propagate additional ROS for an extended amount of time, resulting in higher toxicity.[26]

Increases in ROS generation can cause cellular stress through mitochondrial damage[41] and as a result of damage to the mitochondria there is less energy available for the production of the energy carrier adenosine triphosphate. Considering that there is a dynamic energy budget with a limited resource of energy, when exposed to NMs, energy reserves are used to synthesize more antioxidant defense mechanisms in order to reduce ROS levels as well as keeping vital systems such as heart-beat and swimming occurring. Less energy is diverted to important, yet less vital, processes such as moulting. For example, *D. magna* exposed to 5 and 30 mg L^{-1} of approximately 60 nm TiO_2 NMs showed reduced swimming velocities. This could be due to the extra weight of the NMs causing mechanical stress from the biological coating which also resulted in the inhibition of the second moulting cycle due to reserve energy being used for swimming.[101]

4. Conclusion

Environmental waters contain a wide variety of biological constituents such as proteins, carbohydrates, and NOM, as well as a multitude of small molecules and metabolites, all of which have a tendency to adsorb to the surface of NMs that are deposited into

environmental waters. These biomolecules create an eco-corona which changes the identity of the NM and modulates the NMs interaction with organisms by altering their coating, identity, stability and therefore their toxicity. *D. magna* is a freshwater organism ideal for NMs toxicity testing although there are several amendments that can be made to adapt these protocols to make them more focused and specific for measuring toxicity of NMs instead of bulk materials.

NMs may already have surface coatings when they are deposited into environmental waters, which may get replaced in part or fully by biomolecules existing within the water. There are several NM characteristics that govern the adsorption of biomolecules to NM surfaces including NM size (affecting curvature and surface area), surface charge, and the presence of a coating. The characteristics of the biomolecules also matter, although in general, biomolecules that are higher in concentration adsorb first and then are replaced by those that have a higher affinity for the NM. This new "coating" termed the eco-corona changes the identity of the NM and facilitates the interaction between the NM and organisms and therefore is a modulator of toxicity. The eco-corona can alter the stability of NMs and increases their uptake into organisms such as *D. magna* and therefore increases their toxicity, although this has been shown to be affected by the concentration of biomolecules present in the medium as well as how long NMs have to interact with the biomolecules. The corona also has the ability to evolve once within the organisms, and can be exchanged with other biomolecules released by the microbiome within *D. magna* so that the NMs excreted from *D. magna* may have an entirely different eco-corona from that at the point of entry.

Overall, the presence of biomolecules in the environment creates an eco-corona and must be considered when investigating ecotoxicity effects of NMs. The eco-corona alters the NM identity which can affect the stability of the NMs causing changes in uptake, retention, and excretion toward the organism *D. magna*. Given the static conditions of the OECD toxicity tests which do not represent realistic and the dynamic conditions of the environment, the presence of biomolecules should be incorporated into these tests in order to represent the changes to the NM that may occur in the environment. Such realistic exposure conditions are especially important for systems biology approaches, to ensure that the signaling pathways perturbed by NMs are ecologically relevant ones, rather than reflecting physical damage pathways resulting from “bare” NMs pulling biomolecules from the cellular membranes of organisms to create an eco-corona that would never arise in the environment due to the prevalence of biomolecules to instantaneously coat the NMs.

While the species *D. magna* was used here for illustrative purposes, it is worth noting that the type of organism, its feeding approach, and its microbiome will all play a role in the nature and composition of the eco-corona that forms, and on whether the eco-corona mitigates the NM toxicity or enhances it. Thus, while in human cell culture, toxicity is almost invariability reduced as a result of the reduced surface energy of the NMs surface and the fact that the bound proteins drive the NMs to engage with specific cellular receptors rather than punching holes in the cellular membranes, in the environment this is not always the case as the eco-corona can also alter uptake and retention amounts. What is emerging though is that the same broad rules apply for the eco-corona as for the human protein corona—the affinity of a biomolecule for the specific NM surface drives its attachment strength and durability, and that even very low abundance ecological macromolecules can be concentrated in the NM eco-corona. Additionally, future directions for the investigation of eco-corona, and indeed NMs ecotoxicity, requires understanding of the complete set of biomolecules involved, from the largest natural organic matter fractions through the range of proteins and polysaccharide sizes to the smallest of the small molecules (metabolites), as each play a role in determining the impact of the eco-corona on NMs ecotoxicity.

4.1. Future Perspectives

The investigation of NMs is widespread although toxicity testing under environmentally relevant and realistic conditions is generally lacking, and is an important area of policy that needs to be updated in order to accurately report NM toxicity. Based on the increased understanding of the importance of the eco-corona and the myriad ways in which it can influence the ecotoxicity of NMs, it is vital now to adapt these protocols to enable reproducible corona formation. One approach to achieving this would be by dispersing NMs in pre-conditioned medium allowing NMs to either stabilize or destabilize before re-characterization and exposure to *D. magna* so that protocols include a corona provision. The elegance of this approach is that it can also be applied to other aquatic and sediment/soil dwelling organisms, allowing a consistent approach across ecotoxicity testing. This approach is also consistent with in vitro testing, where NMs are always exposed in serum containing media in order to eliminate non-specific binding effects and membrane damage arising from the highly reactive NMs surfaces.

Determination of the complete corona, including the abundance, coverage, and types of biomolecules of all sizes (proteins, small molecules etc.) forming the corona around different types of NMs would be important future step, recognizing the important roles played by a range of biomolecules. To date the focus has been on proteins (as biologically active agents) and NOM as a dispersant, but the myriad of small molecules (metabolites) present in biological organisms, many of which interact directly with proteins, are also likely to play important roles in how organisms process and respond to NMs. The first efforts to develop a methodology to characterize the small-molecule corona secreted by *D. magna* have recently been published,[102] and further studies are needed to understand the implications of the small molecule corona. Indeed, the ultimate goal is to understand the complete corona—the composition and interplay of all constituents in order to understand the true identity of NMs being exposed to organisms. This would be variable under environmental conditions where multiple organisms exist, although determination of the complete corona based on the secretions of one organism under controlled conditions will still be an excellent step toward determining the composition of the corona as well as the driving factors of toxicity.

As data regarding the composition and evolution of the eco-corona emerge, and its correlation with NMs toxicity is elucidated, the integrated datasets may, in the future, enable the

development of predictive artificial intelligence and/or machine learning models to predict NMs eco-coronas and ecotoxicity, as well as allowing design of NMs with reduced eco-toxicity and/or optimized environmental distribution behaviors based on selective binding of biomolecules, as part of a strategy to develop environmentally benign-by-design NMs. Integration of systems biology approaches and understanding of perturbed signaling pathways under realistic exposure conditions, and identification of key molecular initiating events leading to adverse outcome pathways may also be facilitated through deeper understanding of eco-corona formation, evolution, and exchange.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

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